A STIMULATORY EFFECT OF INSULIN ON PHOSPHORYLATION OF A PEPTIDE IN SARCOLEMMA-ENRICHED MEMBRANE PREPARATION FROM RAT SKELETAL MUSCLE

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1. Introduction

It has been well established that insulin stimulates glycogen and lipid synthesis by influencing protein phosphorylation of some of the enzymes involved in these processes. Three different mechanisms for the insulin effect on phosphorylation of enzymes in the cytosol have been proposed. These involve:

(i) Decreased cyclic AMP-dependent protein kinase activity [1,2].

(ii) Inhibition of a newly discovered protein kinase which is independent of cyclic AMP (3).

(iii) Activation of protein phosphatase [4]. An insulin effect by any of these mechanisms would lead to dephosphorylation of certain metabolic enzymes.

On the other hand we have previously demonstrated that insulin promotes increased turnover of ^{32}P in γ-phosphate of ATP in muscle [5]. In light of these observations we have now investigated the possibility that insulin might influence protein phosphorylations at the level of the plasma membrane. For this purpose we have studied a sarcolemma enriched membrane preparation from rat skeletal muscle. This membrane preparation has been isolated by a mild procedure where treatment with high salt concentrations has been avoided. Protein phosphorylation of this membrane preparation has been investigated and it has been demonstrated that insulin specifically stimulates phosphorylation of a membrane peptide of lower molecular weight.

2. Methods

2.1. *Preparation and characterization of muscle membranes*

The procedure was done essentially according to Kidwai [6]. Rat hind-limb muscles (6–9 g) were homogenized in 0.25 M sucrose with a high speed Ultra-Turrax homogenizer Type TP. 18-19. The homogenate was subjected to filtration by the equipment described by Kidwai and centrifuged at 120 000 × *g* for 60 min. The pellet was suspended in 0.25 M sucrose and subjected to centrifugation on a continuous sucrose gradient. Three different bands were separated on the gradient and characterized by enzyme markers. As shown in table 1 the lighter density membranes (F_1) exerted high activities of adenylate cyclase, Na^+,K^+–ATPase and 5′-nucleotidase. Electron micrographs of this fraction demonstrated membrane vesicles of different sizes. Mitochondria were almost absent and contractile fibers could not be seen. The enzyme distribution of this membrane fraction is consistent with a sarcolemma enriched preparation. Fraction F_3 is mainly composed of mitochondria according to the enzyme markers and the electronmicrographic picture. The enzyme assays were: Na^+,K^+–ATPase [7], adenylate cyclase [8], 5′-nucleotidase [9], cytochrome *c* oxidase [10].

2.2. *Procedure for phosphorylation of membranes*

Assay for membrane phosphorylation was done

Table 1
Marker enzyme activities of fractions isolated from rat hind limb skeletal muscles

Fraction	Mg^{2+}–ATPase (μmol)	Na^+,K^+–ATPase P_i mg^{-1}. 60 min^{-1}	5′-Nucleotidase	Adenylate cyclase (pmol. mg^{-1}. min^{-1})	Cytochrome *c* oxidase
Homogenate	62 (4)	2 (4)	0.18 (3)	110 (3)	0.19 (3)
F_1	218 (4)	155 (4)	1.60 (3)	1380 (6)	0.61 (3)
F_3	169 (4)	45 (4)	0.35 (3)	0 (3)	14.83 (3)

The fractions were separated by sucrose density-gradient centrifugation as described under Methods

F_1 represents sarcolemma enriched membranes isolated at density 1.10–1.115. F_3 represents mitochondria enriched fraction isolated at density 1.16–1.19. Number of experiments in brackets. Mean values are given

according to Malkinson et al. [11] with some modifications. Three ml membrane suspension (F_1) from the gradient was diluted to 10 ml with 50 mM imidazol buffer, pH 7.4, containing 1 mM EDTA and centrifuged at 60 000 × *g* for 45 min. The pellet was resuspended in 250 μl of the same buffer and aliquots containing 50–80 μg protein were incubated in an assay system containing 20 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM imidazol buffer, pH 7.4, with and without 1 μM cyclic AMP. The total volume was 50 μl. Incubation was done for 10–30 s at 30°C and the reaction was terminated with addition of 25 μl of a solution containing 9% SDS, 30 mM Tris/HCl, pH 7.8 and 3 mM EDTA. The mixture was boiled for 2 min and the proteins solubilized by incubation for 30 min at 37°C after addition of 150 mM dithiothreitol. Aliquots of 20 μl were subjected to polyacrylamide slab-gel electrophoresis according to Fairbanks et al. [12]. The protein bands were visualized by the staining procedure involving Coomassie Blue. The gel was cut into slices of 2.4 mm thickness, decolorized with H_2O_2 and dissolved in Soluene 350. Scintillation fluid, 10 ml, was added and counting performed in a Packard scintillation spectrometer.

In some experiments the protein bands were eluted from the gel and tested for the presence of phosphoserine [13,14] and hydroxylamine labile phosphate bonds [15,16]. Purification of skeletal muscle protein kinase was done according to Walsh et al. [17]. The heat stable inhibitor of protein kinase was prepared according to Appleman et al. [18]. Protein was determined by the Biuret method [19].

Insulin was a highly purified preparation of pig insulin from Nordic Insulin Laboratory. Histone IIA was obtained from Sigma and [γ-^{32}P]ATP ammonium salt (spec. act. 15 Ci/nmol) from the Radiochemical center (Amersham).

3. Results

After gel electrophoresis of the membrane fraction (F_1) and staining of the gel with Coomassie Blue 12 different protein bands were visible (fig.1A). As compared with gel electrophoresis of protein standards of known molecular weights, the molecular weight of the bands varied from 120 000 to 20 000. A broad and strongly coloured protein band of mol. wt 88 000 makes up more than half of the protein staining profile. Two protein bands were markedly ^{32}P-phosphorylated when the membrane fraction had been incubated with [γ-^{32}P]ATP (fig.1B). The molecular weight of these bands could be calculated to 88 000 and 15 000, respectively. In addition 3 bands of intermediate molecular weights were slightly [^{32}P]phosphorylated. Protein phosphorylation of these membranes somewhat differs from phosphorylation of muscle membranes prepared by other procedures [20,21]. The position of the radioactive band 1 corresponds with the position of the strongly coloured protein band of mol. wt 88 000. By treatment with hydroxylamine the ^{32}P-radioactivity of this band was released to an extent of 90%. This suggests the presence of acyl-phosphate groups characteristic of Na^+,K^+–ATPase. The results indicate that this peptide represents the catalytic subunit of Na^+,K^+–ATPase, since molecular weights of the same order of magnitude have been reported for purified preparations of this enzyme [22]. Further characterization of this

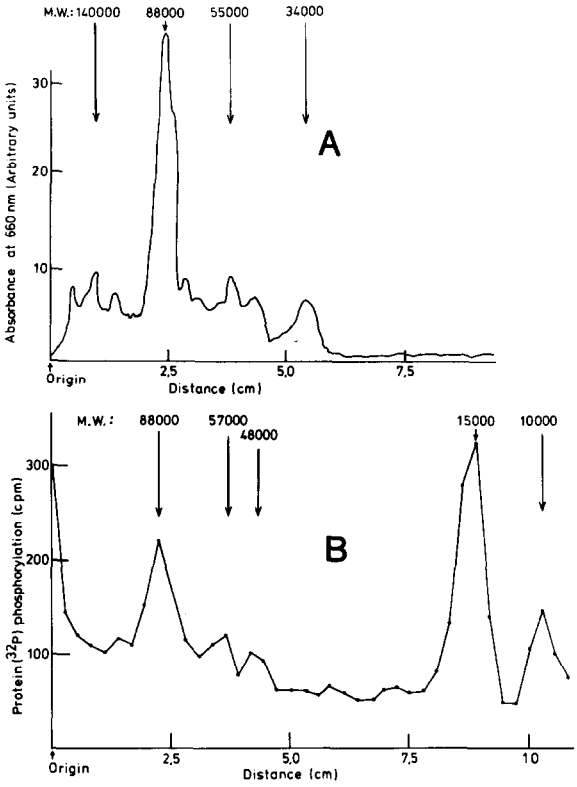

Fig.1A. Protein staining of skeletal muscle sarcolemma membranes. Incubation of the membrane preparation (80 μg protein) was done as described under Methods. The solubilized proteins were subjected to SDS–polyacrylamide gel electrophoresis. After staining of the gel scanning was done at 660 nm by Vitatron Densitometer. Fig.1B. Endogenous protein phosphorylation of skeletal muscle sarcolemma membranes. Membrane suspension (80 μg protein) was incubated with [γ-^{32}P]ATP for 10 s at 30°C as described under Methods. The gel was cut into pieces of 2.4 mm thickness and the radioactivity determined in a liquid scintillation spectrometer.

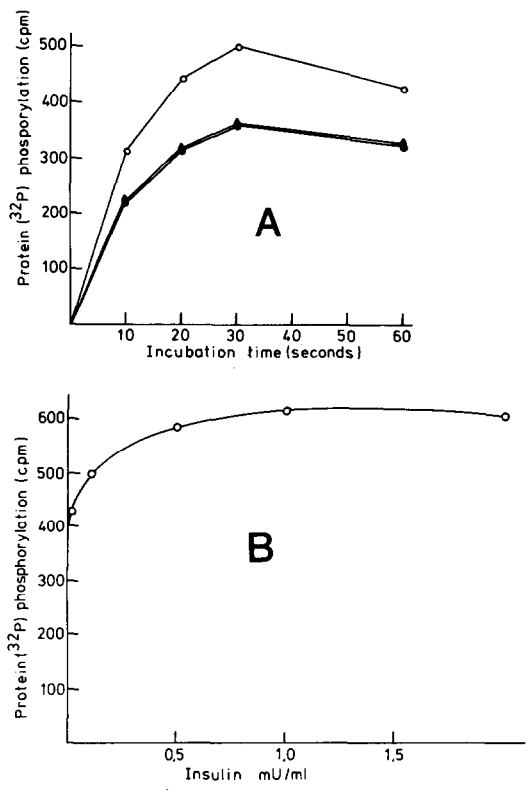

Fig.2A. Time course of ^{32}P-phosphorylation of the endogenous membrane peptide of mol. wt 15 000. Membrane suspension (50 μg protein) was incubated at 30°C at times indicated. (●———●) Incubation of the membranes without additions. (▲———▲) Incubation of the membrane in the presence of 1 μM cyclic AMP. (○———○) Incubation of the membranes in the presence of insulin (1 mU/ml). Fig.2B. The effect of insulin concentration on ^{32}P-phosphorylation of the endogenous membrane peptide of mol. wt 15 000. Incubation of membrane suspension (80 μg protein) was performed for 10 s at 30°C. The curves in A and B represent mean values of three separate experiments.

protein band will be reported elsewhere. By incubation of the membrane with cyclic AMP or with insulin no stimulatory effect of the ^{32}P-phosphorylation of the 88 000 dalton protein band has been observed.

The peptide of mol. wt 15 000 was rapidly phosphorylated when the membrane was incubated with [^{32}P]ATP reaching a maximum value within 30 s at 30°C (fig.2A). Strict linearity was not observed and the phosphorylation slightly declined after 30 s incubation, presumably due to protein phosphatase activity. When the membrane had been incubated with addition of insulin (1 mU/ml) in vitro, phosphorylation of this endogenous membrane peptide was increased to an extent of approximately 40% (table 2). It appears from the time curve of phosphorylation that insulin did not influence the dephosphorylation of the peptide (fig.2A). As shown in table 2 cyclic AMP at a concentration of 1 μM had no effect on the phosphorylation of the endogenous

Table 2
The effect of insulin on ^{32}P-phosphorylation of endogenous membrane protein (mol. wt 15 000)

Group	N		^{32}P-Phosphorylation (cpm)	
Control	5	467	467 ± 17	
Cyclic AMP	5		490 ± 39	
Insulin	5		627 ± 24	(0.01[a])
Insulin + Cyclic AMP	5		612 ± 36	(0.01[a])

[a]Significance of insulin versus control

Sarcolemma membrane suspension (80 μg protein) was incubated with [γ-^{32}P]ATP for 10 s at 30°C as described under Methods

peptide in the absence as well as in the presence of insulin in the incubation medium. Insulin increased the peptide phosphorylation in a dose-dependent manner, half maximal effect being obtained at an insulin concentration of 200 μU/ml (fig.2B). It was shown that ^{32}P-labelling of the peptide is due to the formation of phosphoserine. By elution of the peptide from gel followed by hydrolysis in 6 N HCl for 3 h it was observed that 80% of ^{32}P was released as [^{32}P]phosphoserine.

Phosphorylation of the peptide of mol. wt 15 000 is due to an endogenous protein kinase activity in the membrane preparation dependent upon addition of Mg^{2+}. In the absence of Mg^{2+} in the incubation medium no phosphorylation takes place, half maximal activity being obtained at Mg^{2+} 2 mM. Phosphorylation of the peptide was increased in linear correlation with the amount of membranes (10–100 μg protein). Characterization of protein kinase activity of the membrane preparation is presented in table 3. It is seen that exogenous added histone is phosphorylated by the membrane. Histone phosphorylation is to some extent stimulated by cyclic AMP and markedly inhibited by addition of the heat stable inhibitor protein [23]. This shows that the membrane preparation contains cyclic AMP-dependent protein kinase activity since this enzyme specifically is inhibited by the protein inhibitor [24]. The almost complete inhibition of a purified cyclic AMP-dependent protein kinase by the inhibitor is shown in table 3. However, the inhibitor protein exerts no inhibition of the phosphorylation of the endogenous membrane peptide. Phosphorylation of the membrane peptide of mol. wt 15 000, therefore, is promoted by a kinase distinct from cyclic AMP-dependent protein kinases.

Table 3
The effect of protein kinase inhibitor on protein kinase purified from rat skeletal muscle and on rat muscle membrane protein kinases

Enzyme preparation	Additions	^{32}P-Phosphorylation (cpm)		Inhibition (%)
		−Inhibitor protein	+Inhibitor protein	
Purified protein kinase	Histone	3045	166	95
	Histone + cyclic AMP	8230	307	96
Muscle membranes	Histone	2240	1595	29
	Histone + cyclic AMP	3050	1820	40
Muscle membranes	None	309[a]	336[a]	0

[a]Phosphorylation of endogenous membrane peptide of mol. wt 15 000

The assay of protein kinase was done as described under Methods. Enzyme sources were purified protein kinase (1 μg protein) or sarcolemma membranes (50 μg protein). Histone phosphorylation was assayed by adding 50 μg histone IIA to the incubation mixture and radioactivity determined after separation of histone by SDS–gel electrophoresis as described under Methods. Inhibitor-protein, 30 μg, was present in the assay system when added

4. Discussion

The increased phosphorylation of the endogenous membrane peptide promoted by insulin can be attributed to changes of the molecular structure of the membrane resulting either in increased activity of the kinase or in an increased association of the kinase with the protein substrate. This insulin effect on protein phosphorylation in sarcolemma membranes has not previously been reported. It differs from the insulin effect on protein phosphorylation in fat cells. In these cells it has been demonstrated that insulin specifically increased phosphorylation of a protein of mol. wt 125 000 localized in endoplasmic reticulum [25,26].

Information on insulin effects on plasma membranes occurring as secondary events to hormone–receptor interaction is rather incomplete. Insulin has been shown to inhibit adenylate cyclase in plasma membranes from liver and from fat cell 'ghosts' [27]. Rapid and transitory increase of cyclic GMP promoted by insulin has been reported in liver and adipocytes [28] but not in muscle [29,30]. Insulin has been shown to stimulate efflux of ^{45}Ca from adipocytes and from rat soleus muscle [31]. Phosphorylation of the low molecular weight peptide promoted by insulin, as shown in the present work, may be of regulatory influence on plasma membrane transport processes. Low molecular peptides have been identified as components of ATPases active in ion transport across membranes [32]. It has been indicated that phosphorylation of fat cell membrane peptides of mol. wt 22 000 and 16 000 are involved in insulin regulation of glucose transport [33]. Of particular interest is that an energy-dependent process involving ATP is necessary for the action of insulin on sugar transport in adipocytes [34,35]. The observation made in the present work may be related to this process. It seems likely that the increased phosphorylation of the muscle membrane peptide promoted by insulin may be of physiological importance in mediating insulin stimulation of sugar and ion membrane transport.

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